

# Growth regulation of the rat mandibular condyle and femoral head by transforming growth factor- $\beta_1$ , fibroblast growth factor-2 and insulin-like growth factor-I

Myriam L. Delatte\*, Johannes W. Von den Hoff\*\*, Servaas J. A. M. Nottet\*\*, Hugo J. J. De Clerck\* and Anne M. Kuijpers-Jagtman\*\*

\*Department of Orthodontics, Université Catholique de Louvain, Cliniques Universitaires St Luc, Brussels, Belgium and \*\*Department of Orthodontics and Oral Biology, University Medical Centre Nijmegen, the Netherlands

**SUMMARY** The mandibular condyle is a major growth site and is known to adapt to functional factors. Numerous studies have been performed on the effects of growth factors on the metabolism of primary cartilages, but only a few investigations have examined their action on primary and secondary cartilages. Therefore, the purpose of this study was to compare the effects of insulin-like growth factor-I (IGF-I), transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), and fibroblast growth factor-2 (FGF-2) on the growth of secondary cartilage from the mandibular condyle and primary cartilage from the femoral head of new-born rats. In addition, synergy between these growth factors was investigated. The level of glycosaminoglycan (GAG) and DNA synthesis was analysed after 5 days in culture with the growth factors. The effects of TGF- $\beta_1$  and FGF-2 on growth, tissue organization, and the GAG and collagen content were also evaluated.

The stimulation of cell proliferation by the growth factors was higher in the mandibular condyles than in the femoral heads. The content of the matrix components was reduced more by FGF-2 in the mandibular condyles than in the femoral heads. Both TGF- $\beta_1$  and FGF-2 antagonized the stimulatory effects of IGF-I on GAG synthesis in the two types of cartilage. In contrast, the total growth of mandibular condyles was not affected by TGF- $\beta_1$  and FGF-2 while that of femoral heads was strongly reduced. This was mainly due to the inhibition of chondrocyte hypertrophy. These results show that in spite of the extensive effects of growth factors on the metabolism of mandibular condyles, their dimensional growth was not affected.

## Introduction

The importance of the mandibular condyle for craniofacial growth and development has often been discussed. Several theories on craniofacial morphogenesis have been proposed, of which some define the mandibular condyle as an important growth centre (Baume, 1961; van Limborgh, 1972); others consider it as an organ without independent growth potential that is mainly adapting to the function of surrounding structures (Moss and Rankow, 1968; Koski, 1968; Petrovic *et al.*, 1975). However, some studies have also demonstrated that the mandibular condyle possesses both an intrinsic and an adaptive growth capacity (Meikle, 1973; Kantomaa, 1984).

Cartilage of the mandibular condyle is considered as secondary cartilage, while the epiphyseal growth plate, the synchondrosis, and the cartilage of the femoral head are all considered as primary cartilages. These two types of cartilage have a different embryonic origin. Primary cartilages are part of the primary cartilaginous skeleton, while secondary cartilages are defined as chondral tissues that develop independent of the

chondroskeleton and in close association with membranous bones (Beresford, 1981). Primary and secondary cartilages also differ in histological organization and in the composition of the extracellular matrix (ECM) (Takigawa *et al.*, 1984; Copray *et al.*, 1986; Mizoguchi *et al.*, 1990; Ishii *et al.*, 1998). Although few studies have directly compared the growth regulation of primary and secondary cartilages, the general idea is that primary cartilage has a relatively independent growth potential and is more sensitive to hormonal-like factors, while secondary cartilage is more sensitive to functional factors (Coprpy and Duterloo, 1986; Copray *et al.*, 1986; Rønning, 1991).

In recent years, experiments have indicated that growth factors also play an important role in the regulation of the metabolism of the mandibular condyle. Both insulin-like growth factor-I (IGF-I) and transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) were detected in the mandibular condyle of growing rats, and their localization was shown to be dependent on the stage of development (Li *et al.*, 1998). Fibroblast growth factor-2 (FGF-2) has been detected in cartilage of the mandibular

condyle of adult rats (Tajima *et al.*, 1998). In addition, TGF- $\beta_1$ , TGF- $\beta_2$  and TGF- $\beta_3$  have been found in the cartilage of the porcine mandibular condyle throughout post-natal development (Moroco *et al.*, 1997). These growth factors have also been shown to regulate the metabolism of mandibular condyles and of isolated condylar cells *in vitro* (Maor *et al.*, 1993, 1999; Livne *et al.*, 1997; Molteni *et al.*, 1999; Blumenfeld *et al.*, 2000).

IGF-I, TGF- $\beta_1$  and FGF-2 are also expressed within the epiphyseal growth plate, where they seem to regulate the rate of chondrocyte proliferation, matrix protein synthesis, and terminal differentiation (Hill and Logan, 1992a). Several studies have been performed on the effects of these growth factors on isolated chondrocytes from primary cartilage in culture. In general, they stimulated matrix synthesis and proliferation (Ohlsson *et al.*, 1992; Hill and Logan, 1992b; O'Keefe *et al.*, 1994; Van Susante *et al.*, 2000).

Studies on the action of specific growth factors on the two types of cartilage generally show similar effects. However, FGF-2 is found in all zones of the mandibular condyle of 8-week-old rats but only in the upper layers of epiphyseal cartilage during endochondral ossification (Tajima *et al.*, 1998). Moreover, Visnapuu *et al.* (2001) found differences in the distribution of IGF-I receptors in the mandibular condyle and the epiphyseal growth plate of young rats. IGF-I synthesis during growth of the mandibular condyle of young rats is also different from that of rib cartilage (Visnapuu *et al.*, 2002). These findings indicate that growth factors might indeed differentially regulate primary and secondary cartilages. Synergistic effects of growth factors have also been shown for several types of chondrocyte (Hill and Logan, 1992b; Hill *et al.*, 1992; O'Keefe *et al.*, 1994; Rosselot *et al.*, 1994; Böhme *et al.*, 1995).

As only a few studies have directly compared the regulatory effects of growth factors on primary and secondary cartilages, the aim of this investigation was to compare the effects of IGF-I, TGF- $\beta_1$  and FGF-2 on mandibular condyles and femoral heads of new-born rats. In addition, synergy between these growth factors was investigated.

## Materials and methods

### *Rat cartilages*

Breeding groups of rats were kept under normal laboratory conditions and were fed standard rat chow and water *ad libitum*. The experiments were approved by the Board for Animal Experiments of the University Medical Centre Nijmegen.

Cartilages from 63 4-day-old Wistar rats (Harlan, Zeist, the Netherlands) were used. The rats were decapitated and 126 mandibular condyles and 126 femoral heads were dissected under a stereomicroscope.

Throughout this procedure, the tissue was bathed in IMDM medium (Life Technologies, Breda, the Netherlands) supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Life Technologies). After removing the adhering soft tissue and muscles, the mandibular condyles and the proximal femoral heads were cut to a length of approximately 1.5 mm. The mandibular condyles contained only the cartilaginous tissue of the organ and a small amount of calcified cartilage. The femoral heads contained the cartilaginous tissue of the proximal head and part of the growth plate.

### *Tissue culture*

The cartilages were placed in 24-well culture plates (Nunc, Roskilde, Denmark) in 0.5 ml of culture medium. The culture medium consisted of IMDM supplemented with penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml), ascorbic acid (150  $\mu$ g/ml) (Sigma, St Louis, Missouri, USA),  $\beta$ -glycerophosphate (5 mM) (Sigma) and 0.1 per cent bovine serum albumin (Sigma). The culture medium was changed three times per week. The cartilages were cultured at 37°C in a humidified incubator in an atmosphere of 5 per cent CO<sub>2</sub> in air.

In the first experiment, 55 mandibular condyles and 55 femoral heads were cultured with a single growth factor to determine the effects on glycosaminoglycan (GAG) and DNA synthesis after 5 days. Groups of five cartilages were cultured with 0.1, 1 or 10 ng/ml recombinant human TGF- $\beta_1$ , 1, 10 or 100 ng/ml recombinant human FGF-2 or 5, 25 or 100 ng/ml recombinant human IGF-I (Life Technologies).

In the second experiment, the synergistic effects of IGF-I and TGF- $\beta_1$  or IGF-I and FGF-2 on GAG and DNA synthesis were analysed after 5 days in culture. To this end, 35 mandibular condyles and 35 femoral heads were cultured in groups of five with 100 ng/ml IGF-I and the above-mentioned concentrations of TGF- $\beta_1$  or FGF-2.

In the third experiment, three groups of 24 cartilages (12 mandibular condyles and 12 femoral heads) were cultured for 2 weeks to determine the effect of selected growth factors on growth. One group without growth factors served as the control, the second group was cultured with 10 ng/ml TGF- $\beta_1$  and the third with 100 ng/ml FGF-2. Growth was monitored during culture. At the end of culture, six cartilages per group were labelled either with [<sup>35</sup>S]-sulphate or [methyl-<sup>3</sup>H]-thymidine and prepared for histology and autoradiography. The other cartilages were used for biochemical analyses.

### *Biochemical analyses*

The synthesis of GAGs was determined from the incorporation of [<sup>35</sup>S]-sulphate (Amersham, Little

Chalfont, Bucks, UK) into the tissue and the DNA synthesis from the incorporation of [methyl-<sup>3</sup>H]-thymidine (Amersham). In the first and second experiments, the cartilages were labelled with both 5  $\mu$ Ci [<sup>35</sup>S]-sulphate/ml and 2  $\mu$ Ci [methyl-<sup>3</sup>H]-thymidine/ml for 17 hours. After labelling, the cartilages were washed three times with phosphate-buffered saline and digested overnight at 60°C in 550  $\mu$ l of 1 mg/ml papain (Merck, Darmstadt, Germany) in 0.2 M NaCl, 0.1 M NaAc, 0.01 M L-cysteine HCl and 0.05 M EDTA, pH 6. Finally, aliquots of the digests and the label media were diluted in scintillation fluid (Aqua Luma, Lumac-LSC, Groningen, the Netherlands) and counted in a liquid scintillation counter (LKB-Wallac, Turku, Finland). The rate of synthesis was determined as the total incorporation per culture to exclude the variation due to varying amounts of calcified cartilage. GAG and DNA synthesis were expressed as percentages of the control cultures to allow comparison between mandibular condyles and femoral heads.

Aliquots of the digests were also analysed for total sulphated GAG with a spectrophotometric assay using dimethylmethylene blue (Farndale *et al.*, 1986). The hydroxyproline (HYP) content, a measure of the amount of collagen, was determined after alkaline hydrolysis (Huszar *et al.*, 1980). The HYP and GAG contents were determined as total amounts per culture and expressed as percentages of the control cultures.

#### *Histology and autoradiography*

Cartilages were fixed in 4 per cent neutral formaldehyde solution, dehydrated and subsequently embedded in paraffin. Serial sections of 7  $\mu$ m were cut in the sagittal plane for the condyles and along the long axis for the femoral heads. Central sections were stained with haematoxylin and eosin and the von Kossa method.

To visualize cell proliferation, sections of cartilage labelled with 5  $\mu$ Ci [methyl-<sup>3</sup>H]-thymidine were processed for autoradiography. GAG production was visualized after labelling with 10  $\mu$ Ci [<sup>35</sup>S]-sulphate. In short, sections were coated with Ilford Nuclear Emulsion K5 (Ilford Scientific Product, Moberley, UK), stored at 4°C, developed, and stained with haematoxylin and eosin.

#### *Growth measurements*

Standardized photographs were obtained at the start of the third experiment and twice per week to monitor growth. Diapositives were projected on a graphical tablet (Numonics Corporation, Montgomeryville, Pennsylvania, USA) producing a magnification of  $\times 200$ . The outer contours of the cartilages were drawn with a pointer. The calibrated surface areas were determined using Sigmascan image analysis software (Jandel

Scientific, San Rafael, California, USA). The initial surface area of each cartilage was subtracted from the subsequent measurements to obtain net growth. This procedure excluded the variation caused by different amounts of non-growing calcified cartilage present in the tissues.

#### *Statistics*

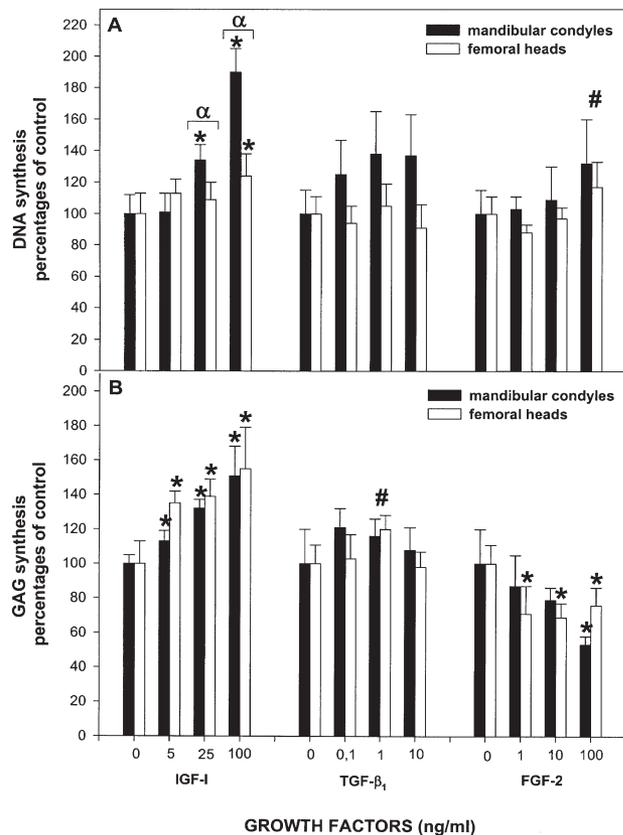
The data were expressed as the means  $\pm$  the standard deviation. For the biochemical variables, differences between the control groups and the experimental groups and between both types of cartilage were tested by two-way ANOVA. In cases of significant effects, Tukey's multiple comparisons test was used for further exploration of the differences. For the growth analyses, differences in the total growth at the end of culture were tested by one-way ANOVA. A *P*-value of less than 0.05 was considered to be statistically significant.

## **Results**

#### *Effects of single growth factors on metabolism*

The DNA and GAG synthesis of the mandibular condyles and the femoral heads were analysed after 5 days in culture with different concentrations of IGF-I, TGF- $\beta_1$  and FGF-2 (Figure 1). The incorporation of [<sup>3</sup>H]-thymidine (DNA synthesis) in control condylar cartilage was  $6.0 \pm 0.7 \times 10^4$  c.p.m. per culture, while that of control femoral cartilage was  $13.2 \pm 1.7 \times 10^4$  c.p.m. (Figure 1a). Both 25 and 100 ng/ml IGF-I significantly increased the DNA synthesis of mandibular condyles to 134 and 190 per cent of the controls, respectively ( $P < 0.05$ ). For femoral cartilage, only 100 ng/ml IGF-I significantly increased the DNA synthesis to 124 per cent of the controls ( $P < 0.05$ ). With both 25 and 100 ng/ml IGF-I, the stimulation was significantly higher for the mandibular condyles than for the femoral heads. With TGF- $\beta_1$ , the DNA synthesis of the mandibular condyles seemed to increase (not significant), while that of the femoral heads did not change. The increase in DNA synthesis observed with 100 ng/ml FGF-2 was significant only if the mandibular condyles and femoral heads were taken together ( $P < 0.05$ ).

The incorporation of [<sup>35</sup>S]-sulphate (GAG synthesis) in the control condyles was  $17.4 \pm 0.9 \times 10^3$  c.p.m. per culture, while that of the control femoral heads was  $60.2 \pm 7.8 \times 10^3$  c.p.m. (Figure 1B). The stimulation of GAG synthesis was significant for both cartilages at all concentrations of IGF-I ( $P < 0.05$ ). No significant differences in GAG synthesis were found between mandibular condyles and femoral heads in response to IGF-I. For cultures with TGF- $\beta_1$  and FGF-2 there was also no significant difference between the two cartilages. Only the groups cultured with 1 ng/ml TGF- $\beta_1$  showed a

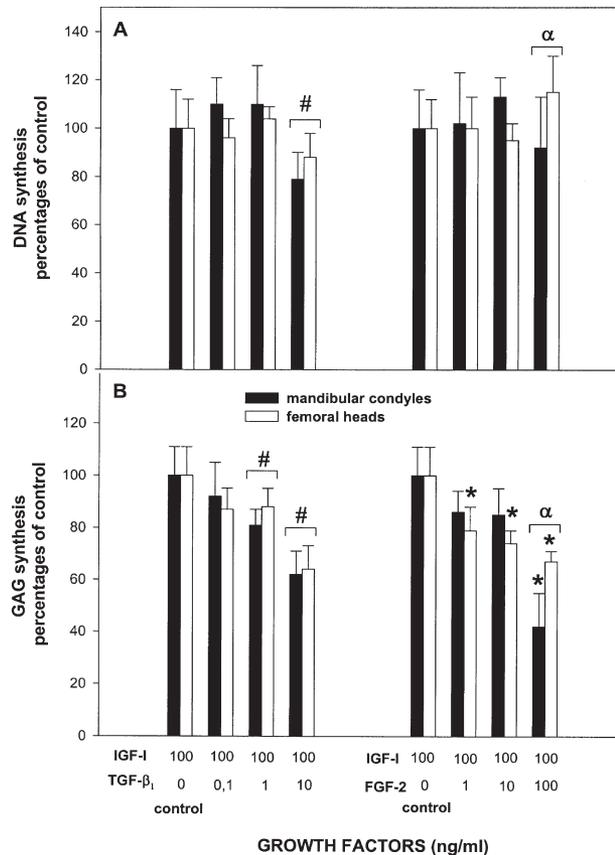


**Figure 1** The effects of insulin-like growth factor-I (IGF-I), transforming growth factor-β<sub>1</sub> (TGF-β<sub>1</sub>) and fibroblast growth factor-2 (FGF-2) on (A) the DNA synthesis and (B) the glycosaminoglycan (GAG) synthesis of the mandibular condyles and femoral heads of 4-day-old rats. DNA and GAG synthesis were analysed after 5 days in culture with the single growth factors in the indicated concentrations. The data represent the mean ± standard deviation of five cultures. The results are expressed as percentages of the control cultures. \* denotes a significant difference ( $P < 0.05$ ) between the control and the treated mandibular condyles or femoral heads. # denotes a significant difference between the control and the treated groups only if the mandibular condyles and the femoral heads are taken together. α denotes a significant difference between the mandibular condyles and the femoral heads (two-way ANOVA followed by the Tukey test).

significant increase in GAG synthesis when the mandibular condyles and femoral heads were taken together ( $P < 0.05$ ). The three concentrations of FGF-2 significantly reduced the GAG synthesis of the femoral heads, while only 100 ng/ml FGF-2 reduced that of the mandibular condyles to 53 per cent of the controls ( $P < 0.05$ ).

#### Effects of combined growth factors on metabolism

To analyse the interactions between growth factors, DNA and GAG synthesis of mandibular condyles and femoral heads were determined after 5 days in culture with 100 ng/ml IGF-I and the indicated concentrations of either TGF-β<sub>1</sub> or FGF-2 (Figure 2).



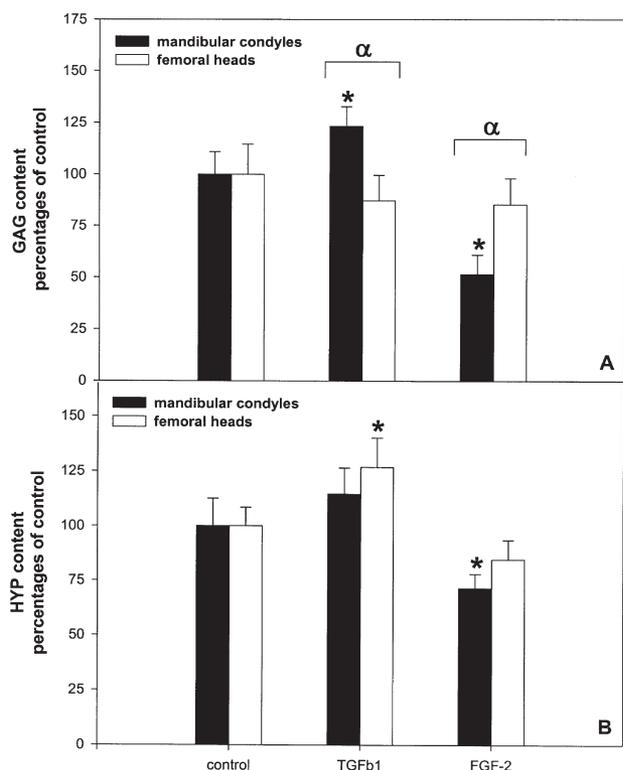
**Figure 2** Synergistic effects of growth factors on (A) the DNA synthesis and (B) the glycosaminoglycan (GAG) synthesis of the mandibular condyles and femoral heads of 4-day-old rats. DNA and GAG synthesis were analysed after 5 days in culture with 100 ng/ml insulin-like growth factor-I (IGF-I; control) and transforming growth factor-β<sub>1</sub> (TGF-β<sub>1</sub>) or fibroblast growth factor-2 (FGF-2) in the indicated concentrations. The data represent the mean ± standard deviation of five cultures. The results are expressed as percentages of the control cultures. \* denotes a significant difference ( $P < 0.05$ ) between the control and the treated mandibular condyles or femoral heads. # denotes a significant difference between the control and the treated groups only if the mandibular condyles and the femoral heads are taken together. α denotes a significant difference between the mandibular condyles and the femoral heads (two-way ANOVA followed by the Tukey test).

*IGF-I and TGF-β<sub>1</sub>*. For both DNA and GAG synthesis, no significant difference between the mandibular condyles and the femoral heads was observed in response to TGF-β<sub>1</sub>. The effects of TGF-β<sub>1</sub> were only significant if both cartilages were taken together ( $P < 0.05$ ). DNA synthesis of all cultures with IGF-I and 10 ng/ml TGF-β<sub>1</sub> was less than that of the controls (with 100 ng/ml IGF alone) and also less than that with 0.1 or 1 ng/ml TGF-β<sub>1</sub> ( $P < 0.05$ ) (Figure 2A). GAG synthesis significantly decreased with the addition of 1 and 10 ng/ml TGF-β<sub>1</sub> compared with the controls cultured with 100 ng/ml IGF-I alone ( $P < 0.05$ ) (Figure 2B). GAG synthesis of cultures with 10 ng/ml TGF-β<sub>1</sub> was also significantly less than that of cultures with lower concentrations of TGF-β<sub>1</sub> ( $P < 0.05$ ).

**IGF-I and FGF-2.** A significant difference in DNA synthesis between the two types of cartilage was found for the cultures with IGF-I and 100 ng/ml FGF-2 ( $P < 0.05$ ) (Figure 2A). GAG synthesis of the mandibular condyles significantly decreased with 100 ng/ml FGF-2 compared with the controls and lower concentrations of FGF-2 ( $P < 0.05$ ) (Figure 2B). For the femoral heads, GAG synthesis significantly decreased for all combinations of IGF-I and FGF-2 compared with the controls ( $P < 0.05$ ). The decrease in GAG synthesis was significantly greater in the mandibular condyles than in the femoral heads in cultures with 100 ng/ml FGF-2 ( $P < 0.05$ ).

#### Effects of TGF- $\beta_1$ and FGF-2 on matrix composition

The GAG and HYP contents of the mandibular condyles and femoral heads was determined after 14 days in culture without growth factor (control), with 10 ng/ml TGF- $\beta_1$  or with 100 ng/ml FGF-2 (Figure 3).



**Figure 3** The effects of transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) and fibroblast growth factor-2 (FGF-2) on (A) the glycosaminoglycan (GAG) content and (B) the hydroxyproline (HYP) content of the mandibular condyles and femoral heads of 4-day-old rats. The contents were analysed after 14 days in culture without growth factor (control), with 10 ng/ml TGF- $\beta_1$  and with 100 ng/ml FGF-2. The data represent the mean  $\pm$  standard deviation of six cultures. The results are expressed as percentages of control cultures. \* denotes a significant difference ( $P < 0.05$ ) between the control and the treated group.  $\alpha$  denotes a significant difference between the mandibular condyles and the femoral heads (two-way ANOVA followed by the Tukey test).

After 2 weeks, the GAG content of the control mandibular condyles was  $33.4 \pm 3.6$   $\mu\text{g}/\text{culture}$  and that of the control femoral heads  $188.1 \pm 27.3$   $\mu\text{g}/\text{culture}$  (Figure 3A). With 10 ng/ml TGF- $\beta_1$ , the GAG content of the mandibular condyles significantly increased to 123 per cent of the controls ( $P < 0.05$ ), while that of the femoral heads did not change. With 100 ng/ml FGF-2, the GAG content of the mandibular condyles decreased to 51 per cent ( $P < 0.05$ ), while that of the femoral heads again did not change. The differences between the relative change in GAG content of the mandibular condyles and the femoral heads in response to both TGF- $\beta_1$  and FGF-2 were significant ( $P < 0.05$ ).

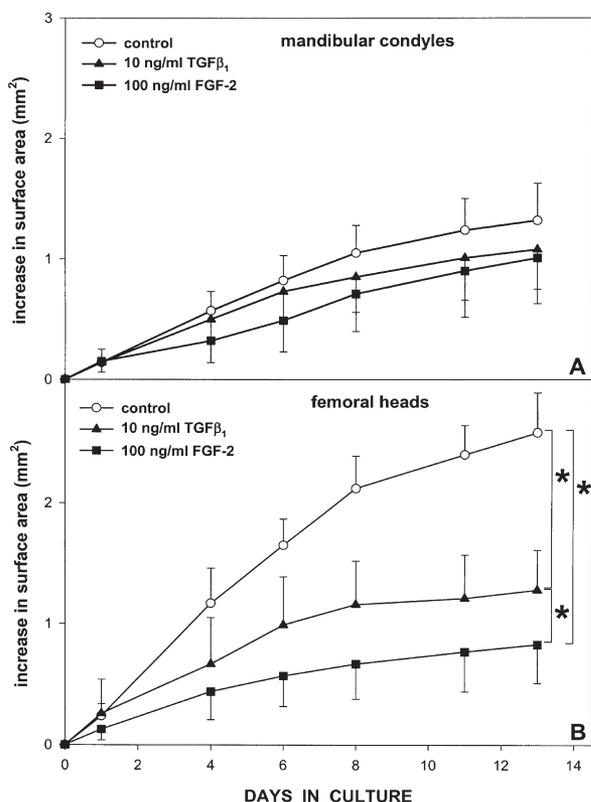
After 2 weeks, the HYP content of the mandibular condyles in the control cultures was  $7.0 \pm 0.9$   $\mu\text{g}/\text{culture}$ , while that of the control femoral heads was  $15.9 \pm 1.3$   $\mu\text{g}/\text{culture}$  (Figure 3B). With TGF- $\beta_1$ , the HYP content of the femoral heads increased to 127 per cent of the controls ( $P < 0.05$ ), while that of the mandibular condyles did not change significantly. In contrast, with FGF-2, the HYP content of the mandibular condyles decreased to 71 per cent of the controls ( $P < 0.05$ ), while that of the femoral heads was not affected.

#### Effects of TGF- $\beta_1$ and FGF-2 on growth

The growth of the mandibular condyles and femoral heads was measured during 2 weeks of culture without growth factors (control), with 10 ng/ml TGF- $\beta_1$  and with 100 ng/ml FGF-2 (Figure 4). The mandibular condyles in the control cultures showed an increase in size of  $1.3 \pm 0.3$   $\text{mm}^2$  in 2 weeks (Figure 4A). TGF- $\beta_1$  and FGF-2 did not significantly affect their growth. The increase in size of the femoral heads in the control group was  $2.6 \pm 0.3$   $\text{mm}^2$  after the 2-week culture period (Figure 4B). With TGF- $\beta_1$  the total increase in size was only  $1.3 \pm 0.3$   $\text{mm}^2$  and with FGF-2  $0.8 \pm 0.3$   $\text{mm}^2$  ( $P < 0.05$ ). Growth was also significantly more reduced by FGF-2 than by TGF- $\beta_1$  ( $P < 0.05$ ).

#### Histology

**Tissue organization.** Initially, the mandibular condyles of 4-day-old rats showed a fibrous and chondroprogenitor layer and a zone of differentiated chondrocytes in the upper half of the condyle, while hypertrophied chondrocytes were present in the lower half of the condyle (Figure 5A). After 14 days in culture, the control mandibular condyles had grown substantially and almost the entire organ consisted of hypertrophied cartilage, while only a thin fibrous, chondroprogenitor and chondrocyte layer remained (Figure 5B). After 14 days in culture with 10 ng/ml TGF- $\beta_1$ , the mandibular condyles had also grown, but the hypertrophied layer was much reduced and occupied only a small part of the organ. A thicker fibrous and chondroprogenitor layer



**Figure 4** Increase in the surface area of (A) the mandibular condyles and (B) the femoral heads of 4-day-old rats cultured without growth factors (control), with 10 ng/ml transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) and with 100 ng/ml fibroblast growth factor-2 (FGF-2). Each group contained 12 cartilages. The values are expressed as means  $\pm$  standard deviation. \* denotes a significant difference ( $P < 0.05$ ) between the groups (one-way ANOVA followed by the Tukey test).

had formed and a wider zone of chondrocytes was present (Figure 5C). With 100 ng/ml FGF-2, the relative proportion of the different layers was similar to that with TGF- $\beta_1$ , but the hypertrophied layer was less reduced (Figure 5D).

Initially, the femoral heads of 4-day-old rats consisted mainly of differentiated chondrocytes (Figure 5E). Hypertrophied chondrocytes were present at the growth plate side of the organ. After 14 days in culture without growth factors, the femoral heads had grown, but still consisted mainly of differentiated chondrocytes. The hypertrophied layer had increased (Figure 5F). With both TGF- $\beta_1$  and FGF-2, the zone of hypertrophied cartilage was much reduced (Figure 5G, H). There was almost no transitional zone between the dividing chondrocytes and the degenerated cartilage. The number of chondrocytes in the femoral heads seemed to be increased by TGF- $\beta_1$  and FGF-2, but the cells seemed smaller.

**Mineralization.** In the mandibular condyles, von Kossa staining indicated mineralization of the ECM in the

hypertrophied layer before culture (Figure 6A). After 2 weeks in culture without growth factors, the degree of mineralization in the mandibular condyles appeared to have increased (Figure 6B). In the mandibular condyles cultured with TGF- $\beta_1$  and FGF-2, there was less mineralization than in the controls (Figure 6C, D).

There was almost no mineralization in the femoral heads before culture (Figure 6E). After 2 weeks of culture, some mineralization was observed in the ECM of hypertrophied chondrocytes and in the degenerated cartilage of control femoral heads (Figure 6F). In femoral heads cultured with 10 ng/ml TGF- $\beta_1$  and 100 ng/ml FGF-2, the staining seemed reduced (Figure 6G, H).

## Discussion

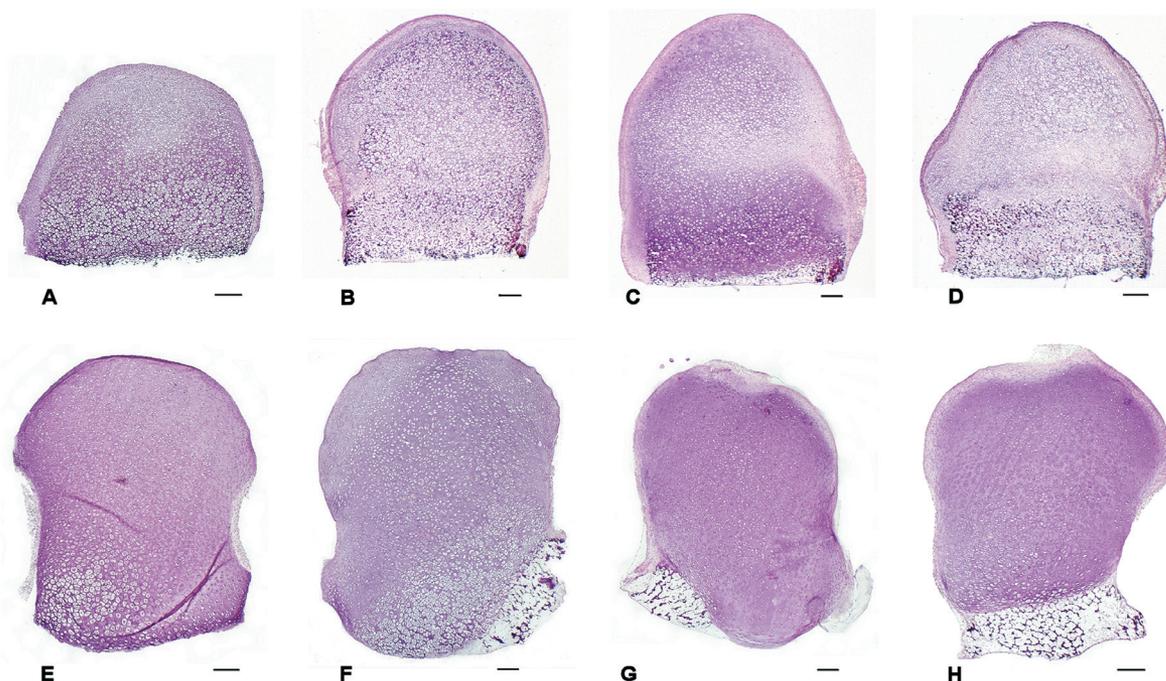
Few reports exist that directly compare the effects of growth factors on primary and secondary cartilages *in vitro*. Conflicting results from the literature may be due to species differences, growth factor dose, the age of the animals or to other differences in experimental conditions. The aim of this study was to compare the effects and interactions of IGF-I, TGF- $\beta_1$  and FGF-2 on the growth of neonatal mandibular condyles and femoral heads *in vitro*.

### Initial differences

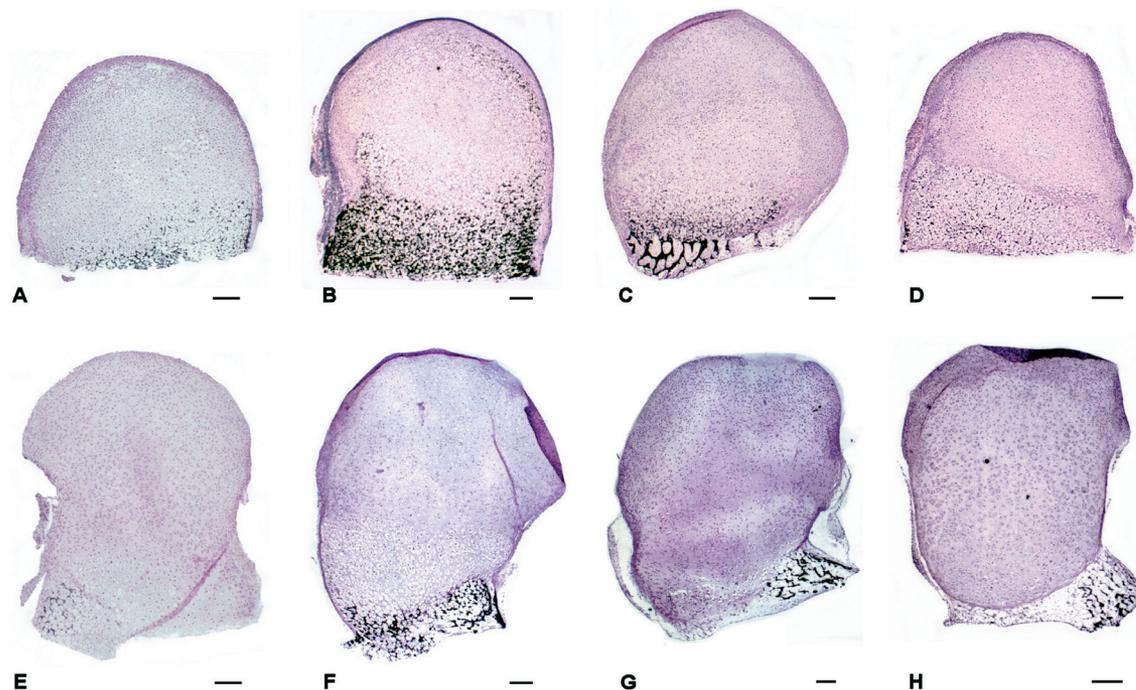
The initial DNA and GAG synthesis rates of the femoral heads were always higher than those of the mandibular condyles. The difference in the rate of cell proliferation can be explained by the different mode of growth of both cartilages. Femoral heads grow by interstitial cell division in the entire organ and mandibular condyles by appositional cell division in the chondroprogenitor layer (Coprav *et al.*, 1986). However, others have recently indicated that it may also occur in the chondroblast layer of new-born animals (Visnapuu *et al.*, 2000). The higher GAG synthesis rate in femoral heads is probably caused by the higher fraction of matrix-synthesizing chondrocytes in femoral cartilage. In addition, there was an increase in matrix per cell.

### Effects of IGF-I

IGF-I is known to be an important regulator of chondrogenesis. In the present study, IGF-I stimulated cell proliferation in both cartilages, although the mandibular condyles were more sensitive. IGF-I has also been reported to increase cell proliferation in the mandibular condyles of new-born and young rats (Maor *et al.*, 1993; Fuentes *et al.*, 2002) and in rat epiphyseal chondrocytes (Ohlsson *et al.*, 1992). Chondrocytes are the proliferating cells in femoral cartilage, while the proliferating cells in mandibular condyles are less-differentiated chondroprogenitor cells. These cells



**Figure 5** Central sections of mandibular condyles (A–D) and femoral heads (E–H) of 4-day-old rats stained with haematoxylin and eosin (bar = 200  $\mu$ m). Initially, the mandibular condyle showed various layers (A). After 14 days of culture, almost the entire mandibular condyle control consisted of hypertrophied chondrocytes (B). After 14 days with transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), the hypertrophied layer was much reduced and there was a wider zone of chondrocytes (C). With fibroblast growth factor-2 (FGF-2), the hypertrophy was less reduced than with TGF- $\beta_1$  (D). Initially, the femoral head consisted mainly of differentiated chondrocytes (E). After 14 days, the hypertrophied layer had increased (F). With TGF- $\beta_1$  and FGF-2, the hypertrophied layer was greatly reduced and the number of chondrocytes seemed increased (G, H).



**Figure 6** Sections of the mandibular condyles (A–D) and femoral heads (E–H) of 4-day-old rats stained with the von Kossa method (bar = 200  $\mu$ m). In the mandibular condyle, the von Kossa staining initially demonstrated mineralization of the extracellular matrix in the hypertrophied layer (A). After 14 days of culture, the degree of mineralization had increased (B). In the 14-day cultures with transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) and fibroblast growth factor-2 (FGF-2), there was less mineralization than in the controls (C, D). In the femoral head, initially there was almost no mineralization (E). After 2 weeks of culture, mineralization was apparent in the degenerated cartilage of the femoral head control (F), while staining was reduced with TGF- $\beta_1$  (G) and FGF-2 (H).

might be more sensitive to IGF-I. Indeed, the type and number of IGF-I receptors on the cells and the amount of IGF-binding proteins in the ECM can modulate the effect of IGF-I (Morales, 1997; Visnapuu *et al.*, 2001). The present results further show a stimulation of GAG synthesis by IGF-I in both the mandibular condyles and the femoral heads. Other studies have also shown that IGF-I stimulates the production of proteoglycans in chicken growth plate chondrocytes (Rosselot *et al.*, 1994), in bovine articular chondrocytes and in ovine growth plate chondrocytes (Sunic *et al.*, 1995). Also, in mandibular condylar cartilage from neonatal rats, IGF-I increased GAG synthesis (Maor *et al.*, 1993).

#### *Effects of TGF- $\beta_1$*

In the present study, TGF- $\beta_1$  increased the GAG content of the mandibular condyles and the collagen content of the femoral heads. However, TGF- $\beta_1$  did not affect the cell proliferation of either cartilage. In other investigations, the addition of TGF- $\beta_1$  to cultures of mandibular condyles from mature mice increased DNA and GAG synthesis (Livne, 1994; Blumenfeld *et al.*, 1997, 2000), whereas a 10-fold lower concentration of TGF- $\beta_1$  inhibited DNA and GAG synthesis (Blumenfeld *et al.*, 1997). These conflicting results on the effects of TGF- $\beta_1$  might be due to a variation in the expression of TGF- $\beta_1$  receptors *in vitro* related to the length of the culture period (van der Kraan *et al.*, 1992). In general, TGF- $\beta_1$  is considered a stimulatory factor of chondrogenesis.

#### *Effects of FGF-2*

In these experiments, FGF-2 decreased both the GAG and collagen contents of the mandibular condyles, but did not affect those of the femoral heads. However, FGF-2 stimulated cell proliferation and decreased GAG synthesis of both cartilages. The latter findings corroborate previous studies on ovine foetal growth plate chondrocytes (Hill and Logan, 1992b), rat rib growth plate chondrocytes (Wroblewski and Edwall-Arvidsson, 1995) and rat mandibular condyles (Molteni *et al.*, 1999; Fuentes *et al.*, 2002).

#### *Interactions of growth factors*

In the present study, 100 ng/ml IGF-I was shown to stimulate cell proliferation in the mandibular condyles more than 10 ng/ml TGF- $\beta_1$  or 100 ng/ml FGF-2. This concentration of IGF-I was also shown to potentiate the effects of other growth factors in growth plate chondrocytes (O'Keefe *et al.*, 1994) and was, therefore, chosen to analyse the interactions of IGF-I with TGF- $\beta_1$  and FGF-2. The results showed that TGF- $\beta_1$  alone did not affect cell proliferation in either cartilage.

In contrast, TGF- $\beta_1$  reduced the stimulation of proliferation induced by IGF-I. A similar interaction was observed in ovine foetal growth plate chondrocytes (Hill and Logan, 1992b). On GAG synthesis, TGF- $\beta_1$  alone had a minor stimulatory effect while it also inhibited the stimulation by IGF-I. Therefore, it is concluded that TGF- $\beta_1$  acts as an antagonist of IGF-I in both mandibular condyles and femoral heads.

In contrast to TGF- $\beta_1$ , FGF-2 did not affect the IGF-I-induced stimulation of proliferation in either cartilage. However, FGF-2 inhibited the stimulation of GAG synthesis observed with IGF-I in both the mandibular condyles and the femoral heads. The FGF-2 effect was dose dependent and was stronger in the mandibular condyles than in the femoral heads. The activity of growth factors can be regulated by exogenous agents such as binding proteins, and by modification of receptor binding (Canalis *et al.*, 1993). TGF- $\beta_1$  and FGF-2 might antagonize IGF-I by stimulation of the production of IGF-binding proteins or by interaction with co-receptors. It has been reported previously that TGF- $\beta$  can stimulate the effect of IGF-I in rat articular chondrocytes via decreased production of IGF-binding proteins or via an effect on the IGF-I receptor (Tsukazaki *et al.*, 1994). In the present study, the combination of growth factors resulted in an inhibition of the IGF-I effect.

#### *Histology*

Histological examination revealed that FGF-2 and TGF- $\beta_1$  strongly reduced the rate of hypertrophy in the femoral heads and to a lesser extent also in the mandibular condyles. FGF-2 has previously been reported to be an inhibitor of chondrocyte terminal differentiation and calcification in isolated rabbit and rat growth plate chondrocytes (Kato and Iwamoto, 1990; Wroblewski and Edwall-Arvidsson, 1995). TGF- $\beta_2$  was also shown to arrest terminal differentiation of isolated chondrocytes in culture (Böhme *et al.*, 1995). However, the present data show that TGF- $\beta_1$  and FGF-2 have no effect on the dimensional growth of mandibular condyles in culture. In contrast, the growth of femoral heads was reduced by both TGF- $\beta_1$  and FGF-2.

#### **Conclusions**

The results show that the metabolism of the mandibular condyles was more sensitive to IGF-I, TGF- $\beta_1$  and FGF-2 than that of the femoral heads. In spite of this, growth of the secondary cartilage of the mandibular condyle was not affected by TGF- $\beta_1$  and FGF-2, while that of the primary cartilage of the femoral head strongly decreased with both growth factors. These findings support the general opinion that the growth of the secondary cartilage of the mandibular condyle is less

sensitive to growth factors than the primary cartilage. Functional factors might indeed be more decisive for the growth of the mandibular condyle.

### Address for correspondence

Anne M. Kuijpers-Jagtman  
Department of Orthodontics and Oral Biology  
University Medical Centre Nijmegen  
PO Box 9101  
6500 HB Nijmegen  
The Netherlands  
Email: a.kuijpers-jagtman@dent.umcn.nl

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